Is it true that plant-derived polyphenols are always beneficial for the human? *In vitro* study on *Leonurus cardiaca* extract properties in the context of the pathogenesis of *Staphylococcus aureus* infections

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The aim of the study was to determine whether *Leonurus cardiaca* L. herb extract (LCE) used at subinhibitory concentration modifies the characteristics of *Staphylococcus aureus*, which is important in the pathogenesis of invasive infections originating from the bloodstream, in a way favourable for the human host. Polyphenol-rich LCE, a common ingredient in pharmaceutical products used for various cardiovascular and nervous system disorders, had shown interesting antibacterial and antibiofilm properties in our previous studies. Our current findings indicate that the following *S. aureus* characteristics decreased, depending on the LCE concentration: (i) formation of aggregates in plasma, (ii) adherence to a fibrin-coated surface, (iii) staphylocoagulase-dependent plasma clotting, (iv) bacterial survival in whole human blood in an *ex vivo* model, (v) expression of cell surface protein A and (vi) synthesis of α-toxin. However, staphylococcal tolerance to exogenous hydrogen peroxide was enhanced after pre-incubation with LCE, possibly due to the increased activity of bacterial antioxidant enzymes, a possibility confirmed by the higher production of superoxide dismutase and slightly higher production of catalase. The use of LCE at sub-MIC in *in vitro* and *ex vivo* models resulted in the weakening of some important staphylococcal immunoprotective attributes but the strengthening of such virulence factors as those responsible for oxidative stress tolerance. Some of these results and the fact that LCE has direct anticoagulant properties, reflected in a reduced thrombin-dependent fibrinogen polymerization rate, suggest a risk of adverse effects, which could be important in the context of *S. aureus* survival in the host.

INTRODUCTION

When bacteria invade the circulatory system, the first line of defence that they encounter comprises numerous humoral immune factors, as well as a range of host cells including phagocytes, platelets, endothelial and other cells. Thus, in order to be successful ‘bloodstream pathogens’, the majority of micro-organisms should be able to interact with immune proteins and soluble receptors or affect the functions of the immunocompetent cells. Septic arthritis, infective endocarditis and sepsis are only three of the serious blood-borne infections frequently caused by *Staphylococcus aureus*. A well-known hallmark of all types of invasive staphylococcal infection is the fact that the bacteria can manipulate the innate and adaptive immune responses. Although slightly different sets of virulence properties are required in the development of different clinical forms of local or systemic staphylococcal infection, the most important role is played by the factors that ensure the survival and multiplication of the bacteria in the bloodstream (Widmer et al., 2006; Edwards & Massey, 2011; Johannesen et al., 2012; Salgado-Pabón et al., 2013; Colavite & Sartori, 2014; Thammavongsa et al., 2015). These bacteria have evolved an abundant range of substances enabling them to evade...
the innate defence mechanism, including strategies utilized by neutrophils, the cells forming the first line of defence. These defences may be subverted at many different stages of pathogenesis: by producing protective capsular polysaccharides or biofilm, secreting specific molecules blocking the phagocyte receptor function, decreasing the efficiency of antimicrobial mechanisms and producing toxins that destroy phagocytes.

After ingestion, *S. aureus* uses even more effective molecular methods to evade the immune system. Surface factors promoting resistance to oxidative killing, catalase (CAT), superoxide dismutase (SOD) and staphylooxanthin, which eliminate the reactive oxygen species used by oxidative-dependent intracellular killing processes, are well known. Moreover, *S. aureus*, like many other bacterial pathogens, generates adenosine to promote its ability to escape phagocytic clearance in the blood and to inhibit neutrophil degranulation, adhesion to vascular surfaces and the oxidative burst (Jongerius et al., 2012; Kim et al., 2012; Thammavongsa et al., 2015). *S. aureus* is able to invade various host cells usually with the participation of the surface MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) family. Recently, the role of secreted factors (SERAMs, secretable expanded repertoire adhesive molecules) has also been reappraised. Among these, coagulase, von Willebrand factor-binding protein and the extracellular fibrinogen-binding proteins (Efbps) are the most prominent in endovascular and other blood-borne diseases (Clements et al., 1999; Cheng et al., 2010; McDow et al., 2012; Powers & Wanderburg, 2014; Dastgheyb et al., 2015; Hall et al., 2015).

Because of the complex relationships between the pathogen and host cells, there is great interest in clarifying these and developing safe methods of intervention. However, the lack of effective antibiotic therapy has resulted in a need to develop new antimicrobial or antivirulence agents, and the use of biologically active products of plant origin is an attractive prospect. Plant-derived substances have been used in folk medicine for a very long time, and some of these have been found to have health-promoting or antimicrobial properties. Phytoalexins, in particular polyphenols, exhibit strong antioxidative, anti-allergic, anti-inflammatory, anticancer, anti-atherosclerotic, antimicrobial and even topically anaesthetic effects (Fraga et al., 2010; Cushnie & Lamb, 2011). As some of these properties directly influence the human immune system, polyphenols also seem to be very important immunomodulators.

It is clear that more in-depth knowledge is required on the effects of their use, their potential adverse interactions with other drugs or their influence on the course of pathological processes such as infection. One substance may alter the bioavailability or the clinical effectiveness of another when two or more are applied concurrently. For example, plant-derived products are known to interfere with the action of anticoagulant drugs. These drugs fall into three groups: inhibitors of clotting factor synthesis (e.g. warfarin), inhibitors of thrombin (e.g. heparin) and antiplatelet drugs (e.g. aspirin). Various herb extracts, decoctions, tinctures or isolated plant-derived supplements that may increase the risk of bleeding when taken, for example with warfarin, include vitamin E, soy-based products, ginkgo biloba, white willow bark, meadowsweet, feverfew, cranberry, fish oil and the omega-3 supplements bromelain, danshen, devil’s claw, dong quai, papain and garlic (Saw et al., 2006; Zuo et al., 2015). In addition, *Leonurus cardiaca* (Lamiaceae) extract (LEC) should be added to this list of herbal products that contain coumarin or its derivatives, with an effect similar to warfarin. The extract from this herb, motherwort, is often compared to those of *Valerianae* radix and *Melissae* folium, which have traditionally been used for a variety of purposes, including cardiac tonic, nerve and an emmenagogue. In Poland, *Leonuri* herba has been used as tea (1.5–2.5 g, two to three times per day) for sedation in nervous system and heart complaints for 30 years. Moreover, various combination products containing *Leonuri* herba are used in a range of forms including herbal tea, powdered herbal substance, ethanol-tincture and ethanolic liquid extract (Committee on Herbal Medicinal Products, 2010; Wojtyniak et al., 2013). However, the use of *L. cardiaca*-derived products is mainly based on traditional indications, as pharmacological studies are limited.

The application and the effects of the products isolated from *Leonurus* spp. have been evaluated from different points of view. As described in the WHO monographs (WHO, 2010), valuable knowledge comes from their use: (i) supported by clinical data, (ii) described in pharmacopoeias and well-established documents and (iii) in traditional medicine, with regard to (i) positive cardiovascular effects that have been reported in open clinical trials; (ii) *Leonuri* herba that is used against cerebral ischaemia, also for the treatment of heart palpitations occurring with anxiety attacks or other nervous disorder; and (iii) traditionally, this herb has been applied for certain types of heart conditions, simple tachycardia and effort syndrome, and specifically for cardiac symptoms associated with neurosis. It has also been used for urine stimulation and for the removal of calculus from kidneys, as well as a remedy for female reproductive disorders. Experimental pharmacology and clinical observation indicate good tissue availability of *Leonurus*-derived extracts and its individual components. Briefly, the results of numerous *in vitro* studies in both cell cultures and animal models confirm cardioprotective, antioxidant and anti-cancer effects of the products. For example, a 70% ethanol extract of motherwort was tested in an open clinical study in patients with cardiovascular disease, and an improvement in cardiac activity and a reduction in blood pressure were observed in 69% of patients. The effect of extract given once daily intravenously for 15 days, which was investigated in 105 patients, improved blood hyperviscosity. Also, oral dosage of a decoction of *Leonuri* herba, given in an open controlled study to 121 normal fertile women, resulted in an increase in intra-uterine pressure in 41.3% of the women. *Leonurus*-derived extracts are also mentioned in a group of plant-origin preparations with the efficacy of photosensitizers topically applied in various skin pathological changes. Thus, every route
of administration – intravenous, oral or topical – is suitable for a therapeutic effect. This undoubtedly confirms the beneficial action of *Leonurus* spp. ingredients. Moreover, the cited monograph mentioned that no information was found about adverse reactions.

Although some *in vitro* studies have demonstrated the biological properties of *Leonurus* extracts and their fractions (Tahmouzi & Ghodsì, 2014), the amount of data, especially on antimicrobial activity, is still insufficient to justify its clinical (*in vivo*) usage. Interestingly, our previous study found that LCE demonstrated previously unknown anti-adhesion and antibiofilm activities (Micota et al., 2014). The present study demonstrates that sub-inhibitory concentrations of LCE possess direct anticoagulant properties that reduce the thrombin-dependent polymerization of fibrinogen. It also confirms that LCE possesses other, previously undescribed, biological activities that may play a role in hypothetical blood-borne infections.

**METHODS**

**Bacterial strains.** *S. aureus* 8325-4 was used as a direct derivative of the NCTC 8325, ATCC 35556 parent strain, with well-defined expression of virulence factors belonging to the group of staphylococcal surface-associated adhesins (MSCRAMMs) and secretable molecules (SERAMs). It had been previously identified in our laboratory as being SpA*, Coa*, Cia*, *α-toxin* and Sak*. Bacteria from frozen stocks were grown for 24 h at 37 °C on tryptic soy agar (TSA) medium (BTL). Then, ready-to-use culture was freshly prepared in tryptic soy broth (TSB; BTL); suspension density was adjusted using spectrophotometry (Densi-La-Meter II; Erba Lachema).

**Preparation of LCE and evaluation of its antimicrobial activity.** Commercially available *L. cardiaca* (motherwort) basic plant material (KAWON-HURT Nowak Sp. j.) was extracted and chemically characterized as previously described by Micota et al. (2014). A stock solution of the lyophilized final polyphenol-enriched LCE was prepared in 50% DMSO and further diluted in liquid medium. The highest concentration of the solvent never exceeded 1.25%, which did not limit bacterial viability. The MIC of the extract was evaluated using the broth microdilution method, according to CLSI (2009). The endpoint (MIC) was defined as the lowest concentration of the extract resulting in a total inhibition of bacterial growth after 24 h incubation at 37 °C compared to the control, as previously described by Micota et al. (2014). Depending on the type of test, LCE was either added to the medium in which the bacteria were cultured for 24 h prior to the preparation of the tested cell suspension or added directly to the bacteria previously cultured in the medium without the extract, for the duration of the experiment. In both methods, LCE was used at a final concentration of 0.5× and 0.75× MIC.

**S. aureus growth kinetics.** *S. aureus* 8325-4 at an initial density of approximately 2×10^7* c.f.u. ml^−1^ in TSB was cultured in the presence of LCE for 3 h at 37 °C. Bacteria in TSB containing 1% DMSO, but without LCE, were used as the control. At indicated time points (0, 0.5, 1, 2 and 3 h), 100 µl of the sample was serially diluted, spread on TSA plates and cultured (24 h at 37 °C) for c.f.u. counting. The results were expressed as the mean number of c.f.u±SD calculated from four replicates of two independent experiments.

**S. aureus survival in whole human blood.** A suspension of approximately 5×10^7* c.f.u. ml^−1^ *S. aureus* 8325-4 in TSB was added at a ratio of 1:4 (total volume 2500 µl) to heparinized blood taken from healthy volunteers following permission provided by KBBN-UL/II/26/2012. Next, LCE was added and the mixture was incubated for 3 h at 37 °C with gentle tube end-to-end rotation. The control consisted of *S. aureus* suspension in blood alone. At indicated time points (0, 0.5, 1, 2 and 3 h), 100 µl of the samples was mixed 1:1 with 2% saponin solution in PBS and kept on ice for 0.5 h to lyse the blood cells. Then, serial dilutions of the samples were spread (100 µl) on TSA and cultured (24 h at 37 °C) for c.f.u. counting. The results were presented as the medians of c.f.u±SD, calculated from four replicates of two independent experiments with one blood donor in each.

**S. aureus coagulase-mediated plasma clotting.** *S. aureus* 8325-4 was cultured for 24 h at 37 °C in the absence or presence of LCE. The cultures were centrifuged at 3000 r.p.m. for 10 min, resuspended in medium free of LCE and adjusted to a density of 1×10^8* c.f.u. ml^−1^ . The suspensions were left at room temperature for 30 min to allow bacteria to ‘rest’ (the time when the bacteria placed in an optimal medium without stressor undergo physiological functions such as *de novo* synthesis of coagulase). Next, 800 µl of the suspension (control or tested) was added to 200 µl rabbit plasma (BioMed). A visual readout of the progressive plasma coagulation was carried out every hour for 1 to 4 h and again after 24 h incubation at 37 °C. *S. aureus* ATCC 29213 (Coa*) and *Staphylococcus epidermidis* ATCC 12228 (Coa−) strains were used as positive and negative controls, respectively, for coagulase release under the described experimental conditions. Two independent experiments were performed, each in duplicate.

**S. aureus α-toxin (Hla) release and protein A (SpA) expression.** *S. aureus* 8325-4 was cultured for 24 h at 37 °C in the absence or presence of LCE. The final concentration of DMSO in control and tested samples did not exceed 1.25%, which did not limit bacterial viability. The cultures were centrifuged at 3000 r.p.m. for 10 min, and supernatants were collected for α-toxin evaluation. The bacterial pellets were used for the assessment of surface protein A (SpA) expression.

The level of α-toxin was determined by standard sandwich ELISA using MaxiSorp (Nunc) microplates and set of antibodies (Ab). Briefly, sheep polyclonal anti-Hla (Abcam) diluted 1:500 in PBS was used as the primary antibodies for the well coating; rabbit monoclonal anti-Hla (Sigma) diluted 1:1000 in PBS/1% BSA was used as the secondary antibodies for the detection of captured HLA, while porcine polyclonal horseradish-peroxidase-conjugated anti-rabbit IgG (Dako) was used as the tertiary antibodies for immune complex detection. The results are presented as ng ml^−1^ of α-toxin released to the culture medium. These values were calculated based on a standard curve of reference Hla (Sigma) (concentration range, 31.3−2000 ng ml^−1^) giving Hla concentration in terms of absorbance measured at λ=450 nm (Victor2 multi-functional microplate reader; Wallac). Two independent experiments were performed, each in quadruplicate.

SpA expression was determined by immunofluorescence assay. Briefly, *S. aureus* suspensions at a density of 1×10^7* c.f.u. ml^−1^ in PBS (400 µl), prepared from bacterial pellets, were mixed with (100 µl) of monoclonal goat anti-SpA FITC-conjugated antibodies (Abcam) diluted 1:100 in PBS/1% BSA. After incubation for 1 h at room temperature, bacterial suspensions were centrifuged at 3000 r.p.m. for 10 min, washed twice with PBS and finally resuspended in 400 µl of PBS. The samples were added (100 µl) to the wells of a 96-well culture plate, and fluorescence was measured at λ=485 nm/535 nm (Victor2 multifunctional microplate reader; Wallac). The results are presented as measured relative fluorescence units. Two independent experiments were performed, each in quadruplicate.

**S. aureus tolerance to H2O2-induced oxidative stress and SOD and CAT activities.** *S. aureus* 8325-4 suspensions were prepared from bacteria cultured for 24 h at 37 °C in TSB without or with LCE. The final concentration of DMSO in control and tested samples did not exceed 1.25%, which did not limit bacterial viability. The cultures were...
centrifuged at 3000 r.p.m. for 10 min, resuspended in the medium free of LCE, adjusted to a density of \(1 \times 10^8\) c.f.u. ml\(^{-1}\) and incubated for 1 h at 37 °C with hydrogen peroxide (\(H_2O_2\)) at a final concentration of 0, 6.25, 12.5 or 25 mM (determined and selected in the preliminary test). Serial dilutions of the samples were prepared (\(10^{-1}\); \(10^{-2}\)), and 5 µl of each was spot inoculated on TSA. The plates were incubated for 24 h at 37 °C. The intensity of bacterial growth was compared to that of control cultures not pre-cultured with LCE and not treated with \(H_2O_2\). Two independent experiments were performed.

For SOD and CAT activity evaluation, the bacterial suspensions prepared as described above were divided into 1 ml samples for the assessment of each enzyme activity and centrifuged at 3600 g for 10 min at room temperature. The cells were washed with 1 ml of PBS and lysed using either 1 ml of 2 % Triton X-100 (Merck) for SOD evaluation or 200 µl of ice-cold assay buffer (included in the test kit) for CAT assessment. Finally, the cell lysates were centrifuged at 14 000 g for 5 min at 4 °C (SOD) or 10 000 g for 15 min at 4 °C (CAT). The enzymatic activity of the lysate supernatants was tested using a superoxide dismutase activity assay kit or a catalase assay kit (Abcam) and presented as recomplement.

Briefly, the enzymatic activity was measured using 1 ml of 2 % Triton X-100 (Merck) for SOD evaluation or 200 µl of ice-cold assay buffer (included in the test kit) for CAT assessment. The cell lysates were centrifuged at 14 000 g for 5 min at 4 °C (SOD) or 10 000 g for 15 min at 4 °C (CAT). The enzymatic activity of the lysate supernatants was tested using a superoxide dismutase activity assay kit or a catalase assay kit (Abcam) and presented as recommended by the manufacturer. The above experiments were repeated twice, each in duplicate.

S. aureus agglutination (aggregation) in human plasma. S. aureus 8325-4 suspension at a density of \(1 \times 10^8\) c.f.u. ml\(^{-1}\) in TSB (2800 µl) was vortexed for 2 x 30 s to ensure that all spontaneous clumps were dispersed. Following this, 30 µl LCE in PBS (final concentrations, 0.5 x and 0.75 x MICs) and 150 µl human plasma from the Regional Blood Center in Lodz, Poland (final concentration of 5 %), were added. Bacterial activity in plasma alone was used as the control for testing spontaneous microbial agglutination. The samples were incubated for 3 h at 37 °C in static conditions; every 30 min, 100 µl of the sample top layer was collected and the absorbance was measured at \(A_{\text{max}}\). The results obtained from two independent experiments performed in triplicate were expressed as the percentage of microbial aggregation (% of SD) in comparison to the control, considered as 100 %.

In parallel, an additional experiment was conducted to check whether LCE alone induced the aggregation of bacteria in the absence of plasma. For this purpose, a bacterial suspension prepared as described above was stained with SYTO9 fluorescence stain (Molecular Probes) for 15 min at room temperature in the dark. Following this, the bacteria were rinsed three times with PBS and resuspended in the same PBS volume. After 3 h incubation of the bacterial suspension with or without LCE, a sample was taken from the bottom of each tube (10 µl) and analysed microscopically (Fluorescent microscope Axio Scope.A1; Zeiss) at a magnification of \(\times 400\). The size and number of bacterial aggregates present in 20 randomly selected locations were compared to those of the control.

LCE impact on thrombin-induced fibrinogen polymerization and S. aureus adherence to the fibrin network. Human thrombin (Sigma) 0.75 U ml\(^{-1}\) in 50 mM Tris-buffered saline (TBS; Dako) was pre-incubated with LCE at concentrations 1, 10, 100, 1000, 3000 and 4500 µg ml\(^{-1}\) for 10 min at 37 °C. Fibrinogen (Sigma) at a concentration of 3 mg ml\(^{-1}\) in TBS, LCE-treated or control thrombin and CaCl\(_2\) 15 mM in TBS were added in triplicate to the wells of a 96-well polystyrene culture microplate (Nunc) at a ratio of 1:1:1:1 (final volume 300 µl). The fibrinogen polymerization potential was monitored every 5 s for 15 min at 37 °C, using a microtitre plate reader (BioRad 550), and expressed as a change of turbidity at 405 nm. The maximal velocity of fibrinogen polymerization (\(V_{\text{max}}\)) and the maximal absorbance (\(A_{\text{max}}\)) were recorded for each absorbance curve. The results obtained from two independent experiments, both in triplicate, were expressed as the medians of maximal velocity percentages (\(V_{\text{max}}\) SD in comparison to the control (100 %)) and as the medians of absorbance (\(A_{\text{max}}\) SD).

The adherence of S. aureus to the fibrin network was evaluated using 96-well microplates coated with a fibrin net consisting of polymerized fibrinogen, prepared as described above, or to the wells of uncoated control plates. After fibrin network formation, control and tested wells were blocked for 2 h at room temperature by 2 % BSA (Sigma) in PBS (250 µl well\(^{-1}\)). Then, the blocking buffer was removed, and suspension of S. aureus 8325-4 at a density of \(1 \times 10^8\) c.f.u. ml\(^{-1}\) in TSB and LCE at 0.5 x and 0.75 x MIC were added to the wells (100 µl of each component). The control consisted of bacterial suspension and TSB with 2 % DMSO. The plates were incubated for 2 h at 37 °C, and then the wells were washed with 200 µl PBS including Ca\(^{2+}\) and Mg\(^{2+}\) (CytoGen) and stained with Alamar Blue (AB; Invitrogen), according to the manufacturer’s recommendations. Sample absorbance was determined at 550 and 600 nm using a multifunctional microplate reader (Victor2; Wallac). The bacterial adhesion (calculated as a percentage of AB reduction) in the presence of LCE was compared to adherence in the control, considered as 100 %. The experiment was performed twice in quadruplicate for each variant.

Bacterial adherence to the fibrin net was also evaluated microscopically using Lab-Tek II glass-plastic chambers (Nunc). Fibrinogen polymerization and bacterial adhesion were prepared as described above. Finally, the fibrin was stained with 100 µl Meyer haematoxylin (Aqua-Med) for 10 min, and the adherent bacteria were stained with 100 µl of crystal violet (for Gram staining; Chempob) for 3 min. Between the applications of these two dyes, the chambers were washed twice with 300 µl of PBS +Ca\(^{3+}\) and Mg\(^{2+}\). Excess crystal violet was removed by washing with PBS and air drying, and the slides were then evaluated microscopically at magnification \(\times 1000\) (Light microscope Nikon YS100).

Statistical analysis. All data were analysed using the STATISTICA 12.5 PL software. Friedman’s ANOVA test was used to determine the significance of the changes over time for related samples. Nonparametric Kruskal–Wallis one-way ANOVA was used to compare differences among the samples from different populations (control, 0.5 x MIC LCE treated and 0.75 x MIC LCE treated). P<0.05 was considered significant.

RESULTS AND DISCUSSION

LCE was investigated chemically in a previous work (Micota et al., 2014). It was found to contain high levels of various classes of polyphenols, which are known to exhibit activity towards haemostatic processes and some of them are known to exert an influence on the course of various infections. Because preparations that can disarm pathogens are an alternative to those that can kill bacteria or inhibit their growth (Khodaverdian et al., 2013; Zhang et al., 2014; Heras et al., 2015), this study used sub-inhibitory concentrations (sub-MICs) of LCE. Since the MIC of LCE was previously established in our laboratory as 6 mg ml\(^{-1}\), concentrations of 3.0 or 4.5 mg ml\(^{-1}\), corresponding respectively to 0.5 x or 0.75 x MIC, were used.

S. aureus survival in whole human blood

It is known that to survive in blood, S. aureus must avoid a variety of immune response mechanisms such as phagocytes, antimicrobial peptides, complement, antibodies and platelet trap action. Our findings demonstrate that LCE is able to modify some pathogenic properties of S. aureus in vitro, resulting in a decrease of bacterial agglutination in...
plasma and reduced microbial adherence to the deposited fibrin network. Also, staphylocoagulase-dependent plasma clotting and bacterial survival in whole human blood were reduced. All of these effects are described in detail below. To confirm these observations, several control tests were performed to check whether the LCE used at sub-MIC does not limit bacterial growth. No statistically significant differences in c.f.u. numbers between control and tested S. aureus cultures in the medium during 3 h incubation were noted at individual measuring points. The density of the inoculum in the control and test samples rose uniformly from an initial value of 2.8±0.2×10^7 c.f.u. ml⁻¹ until the end of the incubation period. The density of S. aureus cultured in TSB free of LCE reached 112.5±12.02 c.f.u. ml⁻¹, while the density of bacterial cultures in the presence of LCE was 121.5±7.0 (0.75× MIC) and 127.5±10.6×10^7 c.f.u. ml⁻¹ (0.75× MIC).

In bacterial samples subjected to 3 h co-incubation with fresh heparinized human blood, the number of viable staphylococci was reduced by the presence of LCE more efficiently than in the control (Fig. 1). However, in the first period of co-incubation, the impact of LCE on bacterial survival was strongly dependent on the extract concentration. LCE at 0.75× MIC enhanced the antibacterial activity of the cells and humoral factors of blood from the very beginning, while LCE at 0.5× MIC slowed S. aureus elimination up to 2 h incubation. Nevertheless, by the end of incubation period the density of S. aureus inoculum in the control (blood alone) had decreased from 95.0±46.9×10^6 c.f.u. ml⁻¹ to 16.3±7.4×10^6 c.f.u. ml⁻¹ (Friedman’s test, P=0.049), while the density of bacterial cultures in the presence of LCE at 0.5× MIC and 0.75× MIC declined to 10.9±6.5×10^6 c.f.u. ml⁻¹ (Friedman’s test, P=0.031) and 9.2±5.5×10^6 c.f.u. ml⁻¹ (Friedman’s test, P=0.010), respectively, although observed differences between the samples treated with LCE and control were not statistically significant at each time point.

**Staphylococcal tolerance to oxidative stress**

S. aureus pre-incubated with LCE later exhibited an increased defensive reaction against oxidative stress, since a more rapid and intensive growth of such bacteria on solid media after H₂O₂ treatment was noted (Fig. 2). This could not have been caused by the direct antioxidant effects of LCE because the bacteria had been washed and resuspended in a medium free of LCE following their previous culturing in the presence of the extract. To determine the cause of this observed phenomenon, the next stage of the study consisted of an analysis of the activity of bacterial antioxidant enzymes. It is well known that to combat such defences as phagocyte-derived reactive oxygen species, S. aureus uses a number of protective molecules such as CAT, SODs (A/M), glutathione peroxidases, globins, peroxiredoxins and staphyloxanthin (Clements et al., 1999; Battistoni, 2003; Gaupp et al., 2012; Painter et al., 2015). In this study, intracellular SOD activity was determined in the lysates of S. aureus cells untreated (control) or treated with sub-MIC of LCE. SOD assessment is based on the reduction of WST-1 substrate to a water-soluble formazan dye by a superoxide anion formed in situ by xanthine oxidase activity, which can be inhibited by external SOD. The obtained results are presented in Table 1. The percentage inhibition of formazan formation (compared to the control) was enhanced by 15.4% and 35.2% in the lysates of bacteria pre-incubated with LCE used at 0.5× and 0.75× MIC, respectively. Although significant differences were observed only for LCE at 0.75× MIC (P<0.001), motherwort extract would appear to have a concentration-dependent effect on enhancing the activity of SOD.

A commercial CAT assay kit was used to measure intracellular CAT in the lysates. The signal from the unconverted H₂O₂ reacts with an OxirEd probe to produce a colorimetric product whose intensity is inversely proportional to CAT activity. CAT activity was not found to change significantly depending on the LCE concentration used (Table 1). It is suggested that, depending on the concentration, some plant extracts decrease the antioxidant activity of bacterial cellular enzymes or even act as pro-oxidants. It seems that their low concentration in body fluids/tissues, despite being safe for eukaryotic cells and protecting them as antioxidants, can frequently have unpredictable effects against micro-organisms. The presence of phytocompounds at low concentration _in vivo_ can affect phagocytosis and microbial killing as part of the oxidative burst (Halliwell, 2008; Fraga et al., 2010; Ginsburg et al., 2013; Danesi et al., 2014). A similar observation is reported by Camagro et al. (2011), who concluded that a flavanone, quercetin, may in fact

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**Fig. 1.** Survival of _S. aureus_ NCTC 8325-4 in whole human blood in the absence or presence of LCE at 0.5× and 0.75× MIC. Results are shown as the medians of c.f.u.±SD of two independent experiments with one blood donor in each. Friedman’s ANOVA test was used to determine the significance of the changes in inoculum density, and Kruskal–Wallis one-way ANOVA was used to compare differences among the samples at each time point. *Significant differences (P<0.05) between 0.5× and 0.75× MICs.
contribute to staphylococcal pathogenesis during the inflammatory stages of staphylococcal disease by decreasing bacterial uptake by phagocytes.

Based on the above results, it can be assumed that during *S. aureus* survival in the blood, initially, the protective (for bacteria) effect of lower LCE concentration (0.5 × MIC) may result from increased bacterial tolerance to H$_2$O$_2$ (Fig. 2), as well as higher activity of microbial SOD (Table 1). The mechanism of neither phenomenon is not clear, but they suggest higher staphylococcal tolerance to exogenous oxidative stress generated by blood cells. However, there are many components capable of limiting the survival of staphylococci and other micro-organisms in human blood, such as lysozymes, complement proteins, antibodies, phagocytes, effector lymphocytes and even toxic products released from degraded cells, which can vary between the donors. Therefore, further research on a larger number of blood samples seems necessary to provide general conclusions.

**S. aureus** α-toxin (Hla) release and protein A (SpA) expression

At the same time, LCE at 0.5 × MIC affected the expression of *S. aureus* virulence factors such as coagulase activity and release of α-toxin (Hla), to a much lesser degree than 0.75 × MIC LCE and did not affect the expression of protein A (SpA) at all (Figs 3 and 4), whereas significant decreases were observed in α-toxin release (Fig. 3; *P*=0.001) and SpA expression (Fig. 4; *P*=0.014) by *S. aureus* pre-incubated with LCE at 0.75 × MIC.

Among the *S. aureus* exotoxins that possess cytolytic activity, α-toxin is described as an important toxin in pathogenesis, causing osmotic cytolysis of human platelets, monocytes and erythrocytes and enhancing biofilm formation (Anderson et al., 2012). Moreover, because of the ability of α-toxin to initiate platelet activation and aggregation, it has been suggested that it can also modulate the normal function of platelets. SpA not only reduces antibody function and impairs phagocytosis but also plays a role in platelet aggregation (Jongerius et al., 2012; Powers & Wandenburg, 2014; Rozemeijer et al., 2014). Therefore, the reduced expression of SpA and release of α-toxin caused by LCE at an appropriate concentration could be considered beneficial by reducing microbial virulence. It seems that motherwort extract can also indirectly modify the physiological function of platelets, but this requires further study on cell aggregation and activation.

However, none of the above-described LCE inhibitory effects seems to be substantial enough to consider the extract as a new protective preparation against staphylococcal infections. The more so that, as demonstrated, bacterial

**Table 1. Activities of SOD and CAT produced by *S. aureus* NCTC 8325-4 pre-cultured in TSB without or with LCE**

<table>
<thead>
<tr>
<th>LCE (MIC)</th>
<th>SOD activity (inhibition rate %)</th>
<th>CAT activity (unconverted nM H$_2$O$_2$)</th>
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<tr>
<td>C</td>
<td>28.02±0.07</td>
<td>11.00±0.04</td>
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<tr>
<td>0.5</td>
<td>43.41±8.25</td>
<td>11.05±0.05</td>
</tr>
<tr>
<td>0.75</td>
<td>63.19±9.34*</td>
<td>10.53±0.24</td>
</tr>
</tbody>
</table>

The prepared bacterial suspensions were lysed, and enzymatic activity of lysate supernatants was tested. In the SOD activity assay kit, WST-1 substrate reduction to a water-soluble formazan dye by superoxide anion formed *in situ* under xanthine oxidase activity is inhibited in direct proportion to increasing levels of external SOD. Results obtained are presented as percentage of the inhibition of formazan formation. In the CAT assay kit, enzyme activity is inversely proportional to the signal from the unconverted H$_2$O$_2$ (nM) reacted with OxiRed probe to produce a colorimetric product. Two independent experiments, each in duplicate, were prepared. Statistical analysis was performed using the nonparametric Kruskal–Wallis test.

*Significant differences (*P*<0.001) in comparison to the untreated control.
tolerance to exogenous oxidative stress was enhanced after exposure to the extract. These results suggest that in the context of infection derived from the bloodstream, the delicate balance existing between the beneficial and adverse effects of LCE can easily be disrupted. However, the course of infection in vivo may be more complex. For example, of the several known fibrinogen-binding proteins that can promote the aggregation of platelets during infection, Efbp is unique. According to Zhang et al. (2011), Efbp can specifically bind fibrinogen and inhibit the aggregation of platelets, which might aggravate acute infection caused by S. aureus. Moreover, Waller et al. (2013) reported that lipoteichoic acids derived from the S. aureus cell wall inhibit platelet aggregation caused by physiological agonists and reduce thrombus formation, thus weakening the biocide potential of platelets.

Staphylococcal aggregation in human plasma

The final stage of the study examined the ability of staphylococci cultured in an environment containing LCE and human plasma to agglutinate into clusters. The spectrophotometric measurements indicated significant changes in S. aureus agglutination during 3 h incubation in each tested sample (Friedman’s test, \( P<0.002 \) for the control, \( P<0.001 \) for LCE at 0.5 × MIC and \( P<0.001 \) for LCE at 0.75 × MIC). A decreased rate and intensity of bacterial agglutination in plasma with LCE compared to the control in plasma alone are demonstrated in Fig. 5. Significant reduction in bacterial agglutination (\( P<0.05 \)) was observed from the first hour of incubation with LCE. An additional experiment based on microscopic observation of fluorescent-stained bacteria was performed and confirmed that bacteria agglutinate in plasma (Fig. 6c) but not in PBS (Fig. 6a) and not in LCE (Fig. 6b). The results of this study confirmed, however, that LCE has a specific inhibitory effect (Fig. 6d); in its presence in plasma, the size and number of bacterial aggregates were lower than in the control bacteria suspended in plasma alone. Since bacteria-forming aggregates are known to exhibit characteristics similar to biofilm populations (Habber et al., 2012), which are more resistant to eradication than planktonic cells, our findings are very interesting. In addition, it was proved that the concentrations of LCE used in our experiments did not generate artefacts that could negatively influence c.f.u. counts, not only when testing the agglutination level but also while assessing the survival of bacteria in whole human blood. As was suggested by Cushnie & Lamb (2011) that aggregation of bacterial cells should always be considered when interpreting data from experiments in which natural polyphenol/flavonoid-rich preparations are used.

Fibrinogen polymerization and S. aureus adhesion to fibrin

A question arises concerning the significance of these observations in the context of the relationship between bacteria and plant-derived products with suspected therapeutic potential. Our findings indicate that LCE, unfortunately, decreased thrombin-dependent fibrinogen polymerization, which is recognized as an important mechanism used by scavenges against micro-organisms penetrating into the bloodstream. Inhibition of fibrinogen polymerization was significantly directly proportional to LCE concentration (Table 2). LCE used at 0.5 × MIC

![Fig. 3](http://jmm.microbiologyresearch.org/1177/3.png)

**Fig. 3.** Alpha-toxin (Hla) release by S. aureus NCTC 8325-4 pre-cultured in TSB without or with LCE. Hla was evaluated in staphylococcal culture supernatants by a standard sandwich ELISA. The results are expressed as individual direct values (ng ml\(^{-1}\)) and the medians from eight replicates in two independent experiments. Statistical analysis was performed using the nonparametric Kruskal-Wallis test, and significant differences (\( P<0.05 \)) are shown in the graph.

![Fig. 4](http://jmm.microbiologyresearch.org/1177/4.png)

**Fig. 4.** Protein A (SpA) cell expression by S. aureus NCTC 8325-4 pre-cultured in TSB without or with LCE. SpA expression was assessed on bacterial cells by immunofluorescence assay. The results are expressed as individual direct values (relative fluorescence units, r.f.u.) and the medians from eight replicates in two independent experiments. Statistical analysis was performed using the nonparametric Kruskal-Wallis test, and significant differences (\( P<0.05 \)) are shown in the graph.
reduced the process by about 35% \((P<0.01)\), while the extract at 0.75× MIC decreased the polymerization by about 50% \((P<0.001)\). Moreover, LCE used in concentrations as low as 100 µg ml\(^{-1}\) reduced the process by about 25% \((P<0.05)\). A similar observation was made by Bijak et al. (2013) concerning the anti-thrombin effect of polyphenol-rich extracts from black chokeberry and grape seeds. Although LCE demonstrated direct anticoagulant activity, it also had a negative impact on the activity of coagulase, the main staphylococcal virulence factor. The use of LCE resulted in slowing the process of plasma clotting stimulated by \textit{S. aureus}, by about 1 to 2 h. This resulted in a delay in obtaining the final result, i.e. a stable clot, which probably increased the efficiency of the mechanisms of the immune system (McAdow et al., 2012; Loof et al., 2015; Zapotoczna et al., 2015). The conversion of fibrinogen to fibrin by activated thrombin is an innate defence in all vertebrates, which immobilizes microbial invaders and attracts immune cells for phagocytic clearance of bacteria. Therefore, every successful bacterial pathogen must evolve mechanisms for escape from fibrin entrapment and subsequent phagocytosis by infiltrating immune cells. \textit{S. aureus} isolates secrete coagulase and von Willebrand factor-binding protein, which, in association with prothrombin, generate biologically active staphylo-thrombin. As staphylo-thrombin does not cleave other substrates of thrombin, it avoids the activation of clotting and inflammatory factors that commonly accompany fibrin polymerization. Moreover, it is known that \textit{S. aureus} agglutinates with coagulase-derived fibrin cables, which requires clumping factor A and four other sortase-anchored surface proteins, whose immunoglobulin-like domains bind to the γ-chain in fibrinogen/fibrin acting synergistically with both coagulas in protecting staphylococci from phagocytic clearance (McAdow et al., 2012; Loof et al., 2015; Thammavongsa et al., 2015). In this study, the impact of LCE on \textit{S. aureus} adherence to both uncoated and fibrin-coated surfaces was tested. The statistically significant decrease of bacterial adhesion in the presence of motherwort extract at 0.75× MIC was noted \((P<0.01)\) for fibrin-coated plate and \(P<0.001\) for uncoated plate; Figs 7 and 8). Thus, the LCE-induced inhibition of \textit{S. aureus} adhesion to fibrin shown in this study can be

<p>| Table 2. Fibrinogen polymerization in the presence of LCE, evaluated spectrophotometrically |
|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>LCE concentration ((\mu g ml^{-1}))</th>
<th>((V_{\text{max}})) % of control±SD</th>
<th>(A_{\text{max}})±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>–</td>
<td>0.315±0.033</td>
</tr>
<tr>
<td>1</td>
<td>100.43±6.18</td>
<td>0.302±0.021</td>
</tr>
<tr>
<td>10</td>
<td>83.45±9.38</td>
<td>0.279±0.008</td>
</tr>
<tr>
<td>100</td>
<td>76.50±4.30*</td>
<td>0.274±0.012</td>
</tr>
<tr>
<td>0 (control)</td>
<td>–</td>
<td>0.294±0.046</td>
</tr>
<tr>
<td>1000</td>
<td>72.79±6.34*</td>
<td>0.271±0.005</td>
</tr>
<tr>
<td>3000</td>
<td>65.19±8.85**</td>
<td>0.253±0.010</td>
</tr>
<tr>
<td>4500</td>
<td>51.90±11.27*****</td>
<td>0.241±0.006</td>
</tr>
</tbody>
</table>

The results are presented as the medians of maximal velocity percentages \((V_{\text{max}})\)±SD in comparison to the control (100%) and as the medians of absorbance \((A_{\text{max}})\)±SD. The experiment was repeated twice, each in triplicate. Statistical analysis was performed using the nonparametric Kruskal–Wallis test. Significant differences in comparison to the control: \(*P<0.05\), \(**P<0.01\), \(***P<0.001\).
considered advantageous for the host. Our earlier findings demonstrated that LCE also reduces adhesion and biofilm formation of *S. aureus* on a surface coated with extracellular matrix proteins such as fibrinogen, fibronectin or collagen (Micota *et al.*., 2014). Therefore, it is worth conducting detailed research to understand the highly diverse antimicrobial activity of natural plant products. These observations may have different implications in the pathogenesis of various infections with an *S. aureus* aetiology, because it is well known that expression of numerous adhesive compounds whose ligands are extracellular matrix components, and production of invasive enzymes and certain toxins, are not equally important in the clinical forms of infections (McAdow *et al.*, 2011; Colavite & Sartori, 2014; Powers & Waldenburg, 2014; Dastgheyb *et al.*, 2015).

As the reserve of available therapeutic options for the treatment of severe infections is steadily becoming depleted, alternative treatments need to be found. One possible answer is the use of biologically active products of plant origin, not always and not everywhere, but when absolutely necessary. Currently, several studies have suggested that the combination of natural compounds and antibiotics or other drugs represents a new strategy for the development of anti-infection therapies. Also, since virulence-associated surface proteins of various Gram-positive bacteria are covalently anchored to the cell wall by transpeptidases (sortases), these represent another set of promising anti-virulence targets (Zhang *et al.*, 2014; Wang *et al.*, 2015). Several groups of polyphenols are known to be plant-derived sortase inhibitors (Kang *et al.*, 2006; Oh *et al.*, 2006; Camargo *et al.*, 2011).

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**Fig. 7.** *S. aureus* NCTC 8325-4 adhesion to the inert or fibrin-coated and BSA-blocked polystyrene surfaces (96-well culture microplates), in the presence of LCE, evaluated using AB staining. The results are presented as individual percentages of bacterial adhesion in the presence of LCE (0.5× and 0.75× MIC) compared to the control (microbial adhesion in culture medium only, 100%) and as the medians from eight replicates in two independent experiments. Statistical analysis was performed using the nonparametric Kruskal–Wallis test. Significant differences between the samples and the control: *P*<0.05, **P**<0.01, ***P***<0.001.

**Fig. 8.** *S. aureus* NCTC 8325-4 adherence to the fibrin network in the (a) absence and (b) presence of LCE at 0.75× MIC. Fibrin and adherent bacteria in Chamber Slide device were stained with haematoxylin and crystal violet, respectively, and evaluated microscopically at magnification ×1000 (Light microscope Nikon YS100). Microscopic images representative of 10 randomly selected locations.
REFERENCES


